EXHIBIT 1

4.7 Analysis of western blot images in Figure 1 from NIH grant proposal 1R44AG056166-01

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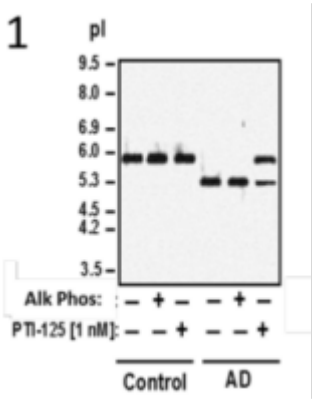
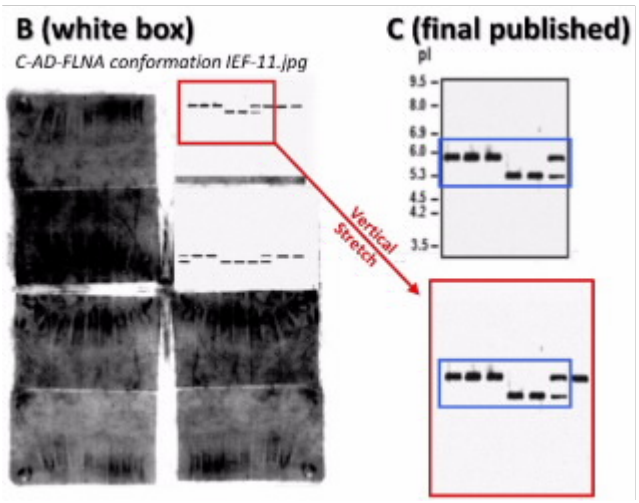
Figure 1 of this proposal (→) purports to show the results of an isoelectric focusing (IEF) western blot experiment to determine properties of filamin A (FLNA). IEF is similar to SDS-PAGE (see document 1), but instead of separating proteins by molecular weight (MW) they are instead separated on a pH gradient. Every protein has an *isoelectric point* (pI), which is the pH value at which the protein has no overall charge. IEF gels *focus* proteins to their pI values on a pH gradient. The processing and development of IEF gels is similar to western blotting of SDS-PAGE gels, involving transfer to a membrane then probing with an antibody. The same image appears in Fig. 2 of Wang *et al.* (2017) *Neurobiology of Aging* **55**, 99-114.



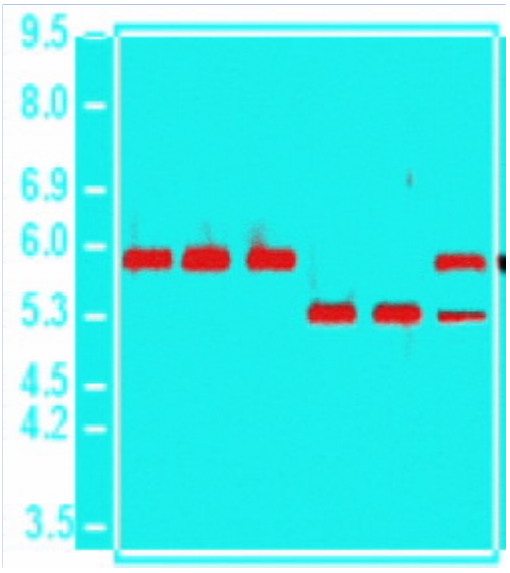
<u>Stage 1.</u> Reverse analysis

This stage asks the following questions: **Q1.** Can the final published image (C) be traced to a white-box image (B) and/or a raw image (A)? **Q2**. Can the final pattern of bands seen in the published image (C) be traced to the source images?

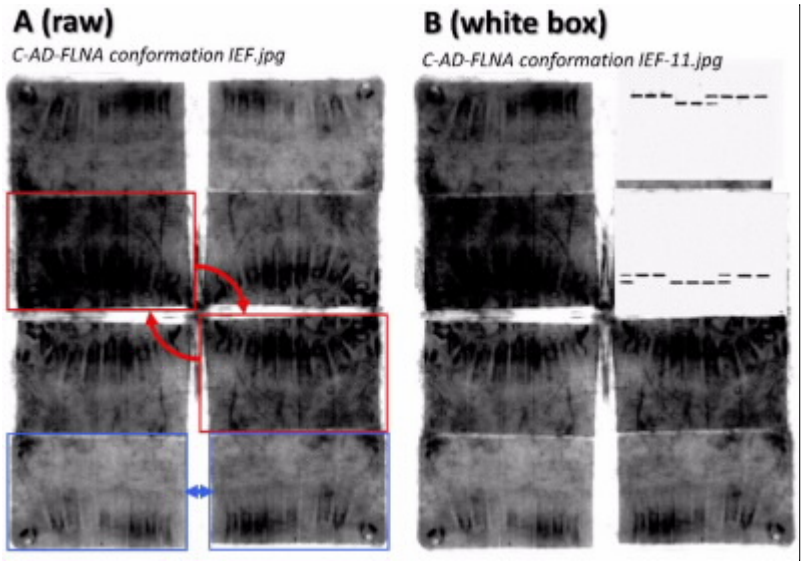
The file "*C-AD-FLNA conformation IEF-11.jpg*" appears to contain an image that is the source for the IEF blot in Figure 1. As shown on the right →, both the bands smudges in the white-box portion of this file (outlined in red) map to features in the published image (highlighted in blue). As such, it is concluded that the white box image (B) *C-AD-FLNA conformation IEF-11.jpg* is the source for the final image (C) in Fig. 1 of the proposal.



This lineage is confirmed → with an overlay of the relevant parts of the two images using the ORI *forensic droplets* in Adobe Photoshop (https://ori.hhs.gov/droplets). Each image is pseudo-colored using a gradient map, with the result that overlapping features appear in red

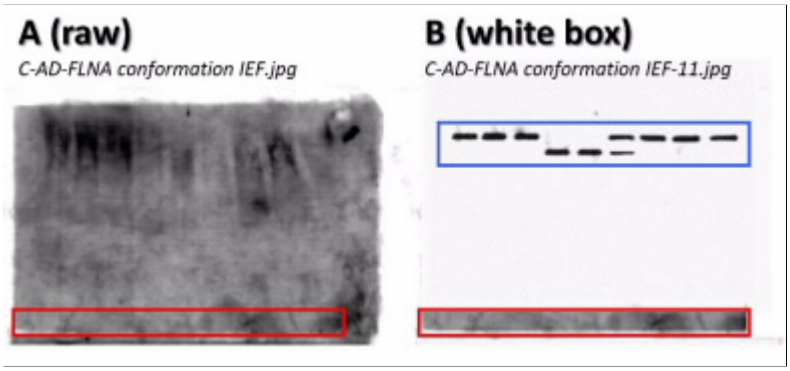


In addition to "*C-AD-FLNA conformation IEF-11.jpg*", an accompanying image file is *"C-AD-FLNA conformation IEF.jpg"*. As shown here → there are several shared features between this raw image (A) and the white-box image (B), indicating they originate from the same piece of film.



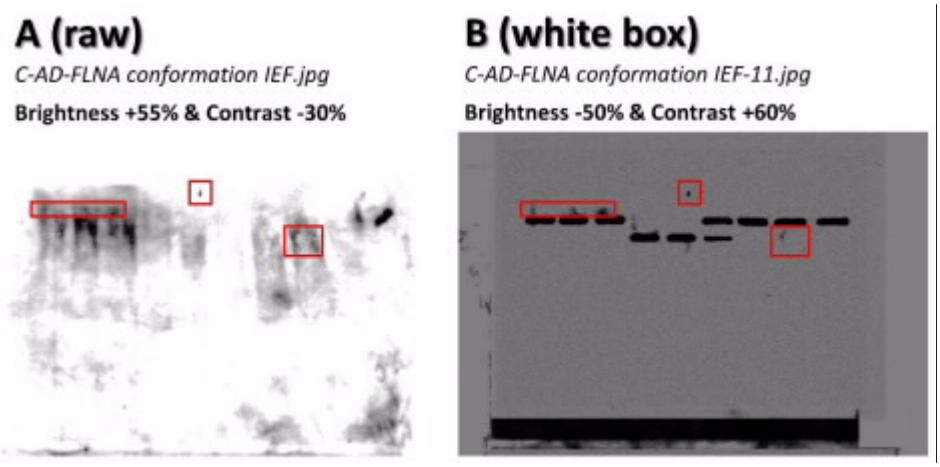
An interesting property of the raw image (A) is that it has both rotational symmetry (red →) and axial symmetry (blue). As such, there were likely only 2 original blots, and the film was exposed 4 times, rotating or flipping it in-between blot exposures. The upper left corner blots are darker, suggesting a longer exposure time.

As shown here →, closer examination of the white-box image (B) and the corresponding area of the raw image (B) reveals some shared noise features (highlighted red). However, the bands in the white box (highlighted blue) cannot be traced to any feature in the raw image (A).

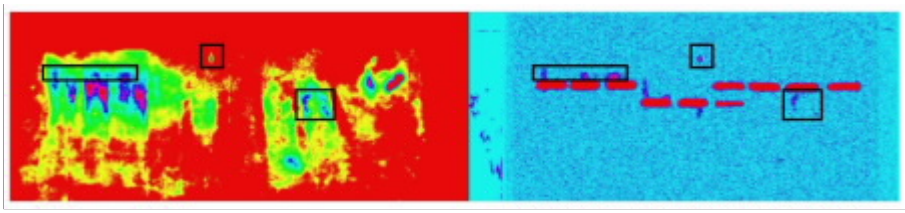


<u>Stage 2.</u> Forward analysis

This stage asks (**Q3**) whether the source images can be manipulated using accepted techniques to recreate the final image? To accomplish this, the raw image (A) "*C-AD-FLNA conformation IEF.jpg*" was subjected to brightness/contrast adjustment, to determine whether features in the white-box image (B) "*C-AD-FLNA conformation IEF-11.jpg*" could be recreated. This manipulation adhered to the fundamental rule of image processing – any adjustment must be applied evenly to the entire field of view. It is unacceptable to adjust selected portions of a blot image such as individual bands.



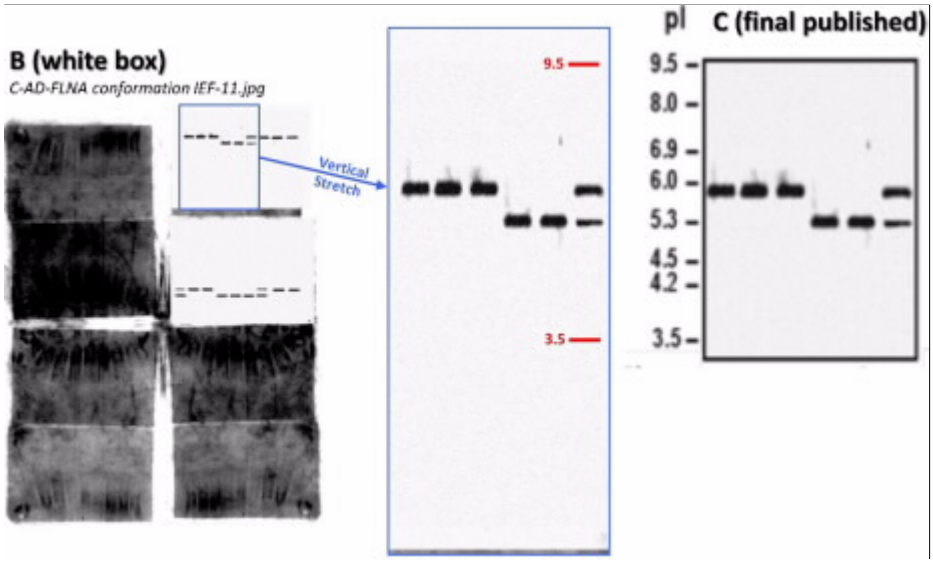
As shown here → enhancing both the raw image and the white-box image did result in some noise patterns above and below the bands being reproduced (highlighted red). This is accentuated further by application of a gradient map → (similar shaped noise highlighted in



black boxes). However, adjustment of the raw image could not cause the bands themselves to appear. As such, the provenance of the bands in the white box image (B) and therefore in the final image (C), cannot be traced to any feature in the raw image (A).

<u>Stage 3.</u> Further anomalies and notable features

A. The pI scale



In comparing the white box image (B) to the final image (C), it is notable that the latter has an isoelectric point (pI) scale alongside the blot. By vertically stretching the white-box image (shown here in blue →) so the size and shape and spacing of the bands matches those seen in the final image, we can use the pI scale on the final image (C) to approximate what the pI scale would have been on the original gel.

According to the methods section of Wang *et al.* (2017) *Neurobiology of Aging* **55**, 99-114, where this image is seenin Fig. 2, proteins were separated on a 3–10 gradient. Thus, the pI range shown here (3.5–9.5, in red) represents almost the complete gradient. This poses a dilemma because <u>the gradient in an IEF gel occupies the entire height of the gel</u>. Here, the full range of the gradient only appears to occupy about 55% of that height. The lowest point on the pI scale (3.5) is slightly more than half-way down the gel. I can think of only three possible explanations for this:

(i) The investigators have invented a completely new type of IEF that does not use the full gel height. There is no scientific rationale for this.

(ii) They ran a gradient that went much lower, and then cut off their scale at 3.5. This contradicts the method published in the paper. Also there no IEF gradient systems that go below a pI of 3 *and* cover up to a pI of 10.

(iii) The pI scale is fabricated. As in the case of regular SDS-PAGE gels discussed elsewhere (see documents 4.1 – 4.6), there are no pI markers on these blots. There is no information on where the pI scale in the final image came from.

B. The feasibility of this experiment.

A fundamental idea principle of the IEF method is that it measures the *native* charge of a protein. Proteins move along the pH gradient within an IEF gel until they find an equilibrium point at which they are no longer charged – the isoelectric point (pI). As noted in Document 1, when regular SDS-PAGE is used to separate proteins by molecular weight, the detergent sodium dodecyl-sulfate (SDS) is added, to give all the proteins a negative charge – this overrides their native charge, so they will run down the gel toward the positive electrode, only being separated by their molecular weight.

control and AD hippocampal synaptosomes were incubated with either 1 nM PTI-125 alone or 1 nM PTI-125 + 10 μM VAKGL ex vivo for 1 hour as described above. Synaptosomes (200 μg) were then sonicated for 10 seconds on ice in 200 μL of modified hypotonic solution (50 mM Tris HCl, pH 8.0, 11.8 mM NaCl, 0.48 mM KCl, 0.13 mM $CaCl_2$, 0.13 mM $MgSO_4$, 2.5 mM $NaHCO_3$, cocktail of protease inhibitors) and treated with 100 μg/mL of alkaline phosphatase at 30 °C for 30 minutes. The reaction was terminated by addition of 100 μM sodium vanadate and 5 mM NaF with cocktail of protein phosphatase inhibitors and solubilized using 0.5% digitonin/0.2% sodium cholate/0.5% NP-40 at 4 °C with end-over-end rotation for 1 hour. Following centrifugation to remove insoluble debris, the obtained lysate was treated with 1% sodium dodecyl sulfate (SDS) for 1 minute to dissociate the FLNA-associated proteins, diluted 10-fold with immunoprecipitation buffer, and immunopurified with immobilized anti-FLNA. The resultant FLNA was eluted using 200 μL antigen-elution buffer (Thermo), neutralized immediately with 100 mM Tris HCl (pH 9.0), diluted to 500 μL with 50 mM Tris HCl, pH 7.5, and passed through a 100 kD cut-off filter to remove low-molecular weight FLNA fragments. Once purified, the FLNA was suspended in 100 μL isoelectric focusing sample buffer. Samples (50 μL) were loaded onto pH 3–10 isoelectric focusing gels and the proteins were fractionated (100 V for 1 hour, 200 V for 1 hour, and 500 V for 30 minutes). The separated proteins were then electrophoretically transferred to nitrocellulose membranes. FLNA was identified by Western blotting with anti-FLNA.

The methods section for the paper where this image also appears (yellow highlight) describes treatment of lysates with 1% SDS. Although FLNA protein was subsequently captured by immuno-precipitation prior to IEF, it is very difficult to remove SDS from proteins. It is generally considered unacceptable to use SDS at any stage prior to IEF, since it imparts a negative charge to proteins that results in poor focusing on IEF gels. Furthermore, it is unclear why immunoprecipitation was necessary for this experiment – if the protein's pI is changing, this should be measurable by loading the protein straight on an IEF gel, without the need to immunoprecipitated it first.

Finally, it is notable that both the raw image (A) and white-box image (B) appear to contain smudges, stains, botches, streaks and other undesirable features within the blot images. These are generally indicative of poor-quality western blots, poor experimental practices, or lack of specificity of the antibody being used. In most laboratories, this type of blot data would be discarded as unusable.

PowerPoint Intermediate File

The .PPT file *"Conformational changes in FLNA-C-AD and PTI-125.ppt"* contains the complete Figure 1 in annotated form, with each of the original images noted above pasted into place and matching the final image. The modification date for this file (2015-01-21) is earlier than the submission of either the paper or the grant. The .PPT file also contains the chart seen in Fig. 1, and extraction of the underlying data to an Excel spreadsheet reveals some anomalies:

(i) The values for the densitometry of the pI 5.2 band in control samples are all either 5 or 10. Also the values for the pI 5.9 band in the AD samples are all 5 or 10. This does not appear to be caused by the densitometry software rounding the values, because all the other densitometry values (in the 1000-1600 range) have randomly distributed terminal digits (e.g., 1542, 1627, 1579) that are not rounded to 5 or zero. While a set of low values is not unusual, if these densitometry values were *real*, one might expect them to be more stochastically distributed (4, 8, 3, 7, 12, 9, etc.) rather than ALL sitting perfectly at 5 or 10. This is highly suspicious, and suggestive of fabrication.

(ii) The figure legend claims N=6, and the data do indeed appear to be calculated based on N=6. However, there are 9 values in the spreadsheet, with the values for the first sample appearing to be replicated 3 further times underneath the 6 values used to make the chart. The purpose of this is unclear but adds confusion to the data set.

(iii) The method used to calculate errors is completely wrong and suggestive of fabrication. Instead of dividing the standard deviation by the square root of N, the St. Dev. has been *multiplied* by either 5 or 2. This results in much larger error bars.

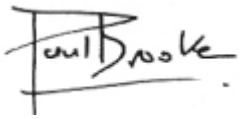
The *original* values are very tightly clustered, so it is likely this would result in unfeasibly small error bars that might be questioned as *too perfect*. Particularly in the case of the two right-most data points on the graph, representing the effect of PTI-125 in AD patients, the values in the chart are 70.3±9.7 and 29.7±7.3, with the error bars clearly visible. The errors here are between 14 and 25% of the mean, so the data appear *noisy*, as expected for the effect of a drug in a complex biological system. However, correct calculation shows that the actual values should be 70.3±2.0 and 29.7±2.0. This would render the error bars so small as to appear biologically infeasible. It is therefore likely that the errors were deliberately manipulated to make the data appear noisier, to avoid scrutiny for being too perfect.

(iv) Regardless the miscalculation of errors, the error bars for the data points elsewhere in the graph are plotted using a set of values that do not appear to originate anywhere in the actual data. They are just pure values in the spreadsheet, not linked to any formula or calculation at all. Notably, in some cases the "errors without an origin" result in an impossible situation. For example, to arrive at an average of 99.7±4%, some of the values contributing to the average MUST have been more than 100%. It is impossible to have more than 100% of a protein in a particular conformation.

As such, it is concluded that the error bars, and potentially the entire densitometry data set, are fabrications.

Summary & Conclusion.

Based on the above evidence, I conclude that the source of the image in Figure 1 of the grant proposal is the white box image (B) "*C-AD-FLNA conformation IEF-11.jpg*". Although this white box image and its corresponding raw image (A) *"C-AD-FLNA conformation IEF.jpg"* share some common features, application of acceptable image manipulation procedures to the raw image was unable to reproduce the bands in the white box image. As such, the provenance of the bands in the final image (C) or the white-box image (B) cannot be traced to the raw image (A). In the absence of original blot images showing the bands of interest as they appear in the final figure, it is my professional opinion that the final figure and its parent white box image and band pattern have been fabricated. Furthermore, extraction of underlying densitometry data from the chart in the PPT file reveals substantial errors in calculation, as well as evidence of fabrication of data. As per the federal definition of scientific misconduct, the presented data do not appear to accurately represent the research record.



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